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ASSESSMENT OF QUALITATIVE AND QUANTITATIVE DATA FROM PATHOLOGICAL HAIRS - A CRITICAL EVALUATION OF SCANNING ELECTRON MICROSCOPE AND PROTON INDUCED X-RAY EMISSION ANALYSES

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Abstract

Analysis of single hair fibres in genetic disorders is a desirable complement to the clinical diagnosis. The scanning electron microscope (SEM) allows detailed study of the surface morphology of hair fibres which may explain some mechanical characteristics of the pathological hair. Quantitative elemental data may indicate biochemical or metabolic abnormalities.

In this preliminary study we assess the feasibility of combining SEM and proton induced X-ray emission (PIXE) analysis on single hair fibres from 12 cases of genetic disease influencing the integument status. We conclude that SEM is a valuable tool in the analysis of hair pathology. The macro-PIXE technique involves some methodological and technical problems which in many cases are likely to be solved by using a proton microbeam. However, this means that routine methods have to be abandoned and careful selection of the material for analysis is an imperative necessity.

KEY WORDS: Pathological hair (fibres), genetic disorders, scanning electron microscopy, proton induced X-ray (micro-)analysis.

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Introduction

The surface structure of a pathological hair may be well described by the use of the scanning electron microscope. Even some mechanical characteristics of such hairs may be visualized after forcing the fibre into a knot (7). In the case of genetic disorders not only the surface and the mechanical characteristics are of interest but also quantitative data on the elemental content with special interest focussed on sulfur. Particle probes may provide such data (1) and generally the electron microprobe is the instrument of choice, as it often allows a scanning electron micrograph to be produced in connection with the actual analysis. With the proton beam only some instruments (9, 10) allow scanning for morphological visualization but several of such instruments are presently under development e.g., in Lund, Sweden. The proton induced X-ray emission (PIXE) analysis furnishes quantitative elemental data of all detectable elements (Z = 14-80) simultaneously and its remarkable sensitivity allows the detection of elements down to a level of $\leq$0.2 ppm when conditions are optimized. By magnetic focussing of the proton beam it may attain a size of approximately 1x1 μm^2 allowing a proton microprobe (PMP) analysis to be performed at subcellular resolution. In comparison to conventional PIXE (with beam diameters in the millimeter range) PMP analysis requires significantly more preparative work before analysis. The sensitivity for microPIXE is however roughly in the same order as that for the macro-PIXE, i.e., <2 ppm.

In a previous study we have provided a 'normal baseline' of sulfur and zinc content in hairs from a normal, healthy caucasian population of both sexes in the age range 10-65 years (3).

In addition to a morphological characterization by scanning electron microscopy (SEM), the present study included PIXE analysis of some of the studied cases. Our main interest has thus been directed towards finding diagnostic parameters in a number of pathological hairs from patients suffering from various genetic disorders. The ultimate aim is to provide the clinicians with additional tools in the diagnosis of (genetic) diseases involving changes in the hair structure and composition.

Materials and Methods

In general the hair samples were collected from an area 1-2 cm above the right ear of the proband by plucking. This allows determination of what part of the hair growth cycle the specimen belonged to, i.e., anagen or telogen phase (3). In some cases it was only possible to obtain cut specimens.

Hair specimens were mounted on aluminium stubs with double-sticking tape without any treatment before mounting. The specimens were gold-sputtered in a Leybold SCD 030 sputtering equipment and viewed in a Philips 505 SEM at 5 kV. This low acceleration voltage diminishes charging of the specimen and still provides optimal resolution at the magnifications used (40-2000 x). Occasionally 30 kV was used. For PIXE analysis conventional non-glassed 5 x 5 cm^2 slide frames for photographic transparencies were used for mounting the hair specimens. This allows automatic PIXE analysis of up to 40 specimens without breaking the vacuum of the PIXE analysis system (6, 8).

The particles forming the proton beam were obtained from an ion source and accelerated in an electrostatic accelerator (PELLETRON) to 2.5 MeV and subsequently focussed onto the target (the hair specimen) by a series of quadrupole magnets providing a beam cross section at the target of 5 mm (diameter). The proton induced X-rays were detected in an energy dispersive detector system (Kevex FWMH 158 eV at 5.9 keV) placed at an angle of 135^o relative to the incident beam (6). Data for all elements heavier than Al (Z=13) were simultaneously stored in a multichannel analyzer (Nuclear Data 6620), transferred to magnetic tape and subsequently subjected to computer analysis. The details of the algorithm used at computation has been given elsewhere (5).

Results

We have chosen to present the SEM findings of the analyzed cases as separate entities in this section of the paper. In addition the results of the PIXE analyses are presented in Table 1.

Case 1

Boy, born 1975. Diagnosis: Haidu-Cheney's syndrome (HCS) (11). Retarded length growth, retarded development of limbs due to skeletal anomalies including a permanently open fontanel. This patient in addition has cystic kidneys. Mental development is normal. The hair fibres are conspicuously stiff and coarse. Mother and maternal aunt have the same type of hair, but little skeletal involvement.

In the SEM a number of morphological variations were discerned ranging from almost normal surface morphology to obviously pathological surface characteristics. The edge-to-edge distance of the cuticle cells appears rather short.

Fig 1: Haidu-Cheney's syndrome hair fibre shows irregular cross-section demonstrated by the knot configuration and a cut end (upper left) (case 1). Bar = 100 µm.

Fig 2: Haidu-Cheney's syndrome (case 2). The irregular cross-section is revealed by the knot configuration. Bar = 100 µm.

Fig 3: Diagnosis osteogenesis imperfecta in a 43 year old woman (case 3). Flat, twisted hair fibre with normal cuticle pattern. Bar = 100 µm.

Fig 4: Same patient as in Fig 3. Spontaneously appearing fracture of hair fibre and defect adhesion of cuticle scales (upper left, lower right). Bar = 100 µm.

The cross section of the investigated hair fibres was irregular (Fig 1).

The PIXE analysis did not indicate any obvious deviation from normal conditions (Table 1). There was good correspondence between the two hairs analyzed, the differences being of the same order as those seen in a normal material (3).

Case 2

Boy, born 1968. Diagnosis: Haidu-Cheney's syndrome (HCS). Previously suspected to be a case of ectodermal dysplasia but patently opened sutures of the skull suggest diagnosis to be HCS. Patently opened sutures and coarse hair structure are also found in the father.

SEM investigation shows irregularities of the hair fibre cross-section along the fibre (Fig 2). The adhesion of the cuticular cells appears to be subnormal. Corresponding properties are found in the father's hair.

PIXE analysis showed the data to be within the normal range.

Case 3

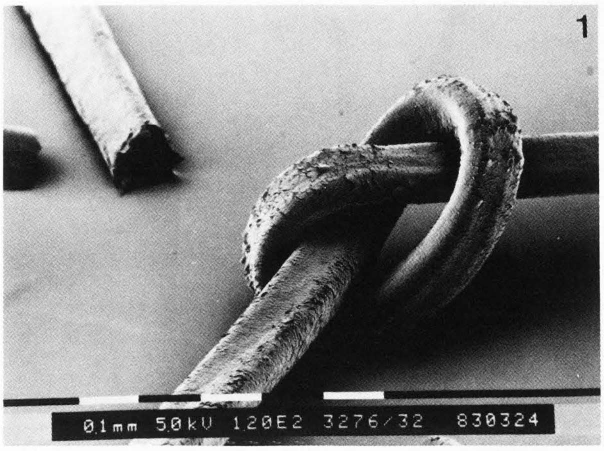
Female, born 1943. Diagnosis of osteogenesis imperfecta. In view of all the spontaneous skeletal fractures this patient has suffered over the years, little attention has been paid to her hair which breaks easily and does not attain a length permitting female coiffure ($\sim$5-7 cm). In the SEM flat, contorted hair fibres were seen (Fig 3), sometimes including clear breaks through cuticle and cortex indicating a conspicuous brittleness (Fig 4).

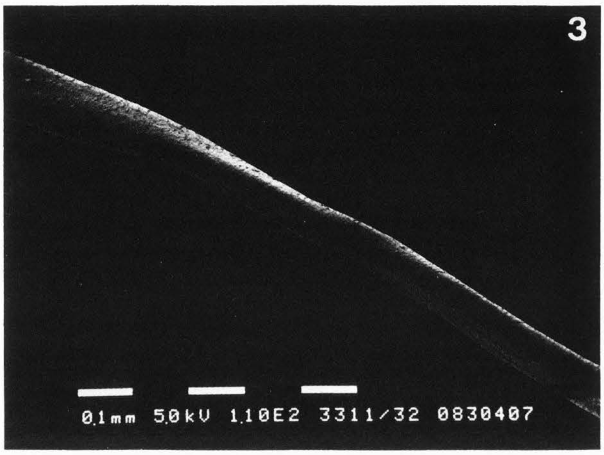
PIXE analysis showed that the elemental composition of the hair investigated lay within the normal range.

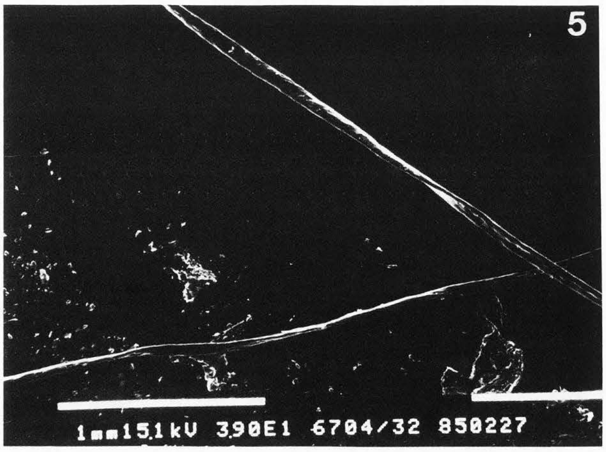
Case 4

Boy, born 1983 with an unsettled diagnosis. Brittle thin hairs. The SEM depicts flat, longitudinally twisted hair fibres (Fig 5). The surface was characterized by small to large depressions and the inherent brittleness of the hair fibres was revealed by the finding of loosened shingles (cuticular cells) (Fig 6) and damaged surface (and internal) structure at kinks (Fig 7).

PIXE analysis: Data lay within the normal range.







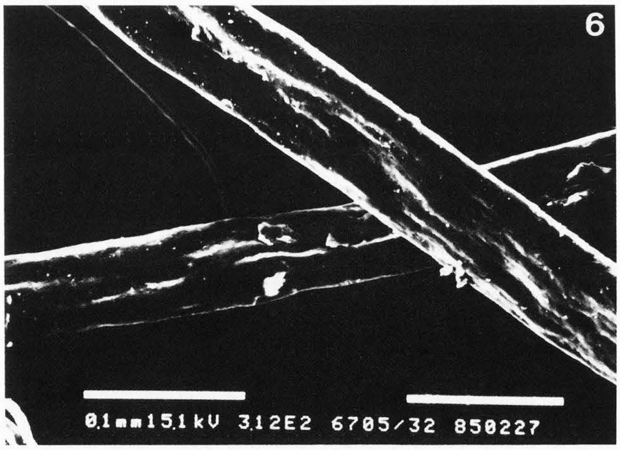


Fig 5: Unsettled diagnosis in a boy, 3 years old (case 4). Note extreme flatness in fibres which have the appearance of collapsed, thin-walled tubes. Bar = 1000 µm.

Fig 6: Same case as in Fig 5. Close up reveals the highly irregular surface configuration of the fibres with barely recognizable cuticular pattern, minor and major grooves, etc. Bar = 100 µm.

Table 1: QUANTITATIVE ELEMENTAL DATA FROM HAIRS IN DISEASE: ELEMENTS IN ppm

Case no	S(%)	Cl	K	Ca	Fe	Cu	Zn	Cu/Zn	Hair mass µg
1/1	3,9	590	28	230	18	21	170	0.124	46
1/2	4.0	800	22	180	13	15	170	0.088	41
2	4.2	170	30	590	4	30	175	0.171	53
3	4.0	650	70	1200	22	20	210	0.095	60
4	3.3	1100	380	480	14	12	150	0.080	53
5	4.6	2800	120	92	17	12	150	0.080	19
6	3.9	2200	55	140	8	33	220	0.150	53
7	4.8	790	42	410	18	180	430	0.428	28
8/1	4.3	780	11	200	12	100	170	0.590	46
8/2	4.3	780	22	210	13	110	170	0.650	46
9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	4.8	1500	230	630	20	29	190	0.152	24
11	3.3	1100	280	680	22	18	110	0.164	53
12/BT	3.3	1300	310	580	17	32	110	0.291	15
12/AT	4.6	870	100	780	21	34	240	0.142	15
Normal Reference Values	4.9[1] 4.2-4.7[2]	3300[1] 750-4805[2]	150-663[2]	330[1] 146-3190[2]	5-67.6[2]	12-27.5[2]	170[1] 99-220[2]		

[1] Ref (3), [2] Ref (4) - the range of values published are given here.
n.d.: not determined.

Fig 7: Same case as in Figs 5 & 6. The spontaneously appearing kink with a depression suggests collapse of the interior of the fibre. Bar = 100 µm.

Fig 8: Girl aged 7 years (case 5) with sparse hair growth and an unsettled diagnosis. The irregular cross-section is demonstrated by the knot configuration. Cuticle pattern appears normal. Bar = 100 µm.

Fig 9: Case 6 with tentative diagnosis hypohidrotic ectodermal dysplasia. Normal anagen root obtained by plucking. Further out on the fibre minor irregularities of the surface appear (upper fibre). Bar = 1000 µm.

Fig 10: Same case (case 6). Close up on fibres reveal minor grooves residing over relatively short distances parallel to the fibre axis. The cuticle pattern does not appear to be disturbed. Bar = 100 µm.

Case 5

Girl, born 1979, unspecified diagnosis, with sparse hair growth since birth. Hair fibres are lost when they reach a length of 5-6 cm and appear to be loosely anchored. Broken hairs are observed at ocular inspection. At SEM some hair fibres have an irregular cross-section much like that found in cases of spun glass hair (2) including longitudinal grooves and angular cross-section (Fig 8).

PIXE analysis: Data lay within the normal range.

Case 6

Girl, born 1964. Tentative diagnosis: hypohidrotic ectodermal dysplasia. Very fair, dry, and brittle hair. In certain areas of the scalp the skin is sclerotic. There are teeth anomalies with apparently thin enamel and hypodontia.

Only a few milimeters out from the characteristic sheath of an apparently normal anagen root (Fig 9) the hair fibre structure shows an irregular surface morphology with minor depression (Fig 10). Often the hair fibres had considerable lengths of a rather flat and sometimes twisted appearance (Fig 11).

PIXE analysis: The data computed lay within the normal range.

Case 7

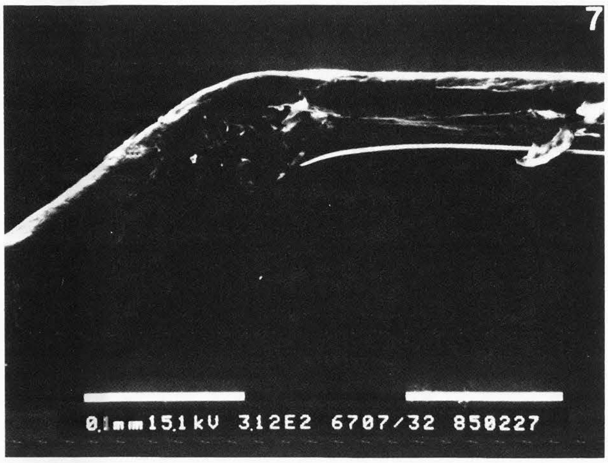
Girl, born 1973. Tentative diagnosis: Epidermolysis bullosa Weber-Cockayne. This girl is deaf in one ear and has a myopia of 2-5 diopters. Sparse hair growth, rough texture of scalp hairs, likened to pubic hairs by the consultant.

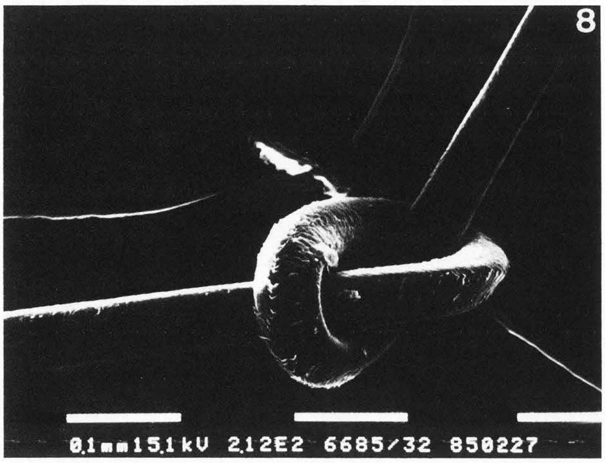
SEM investigation shows that the gross characteristics of these hairs do not conspicuously deviate from those of normal hair. The cross-section of the fibres is rather flat (Fig 12). Surface characteristics appear to be those of a normal hair fibre and there is no correspondence in the SEM to the clinical description of the hair.

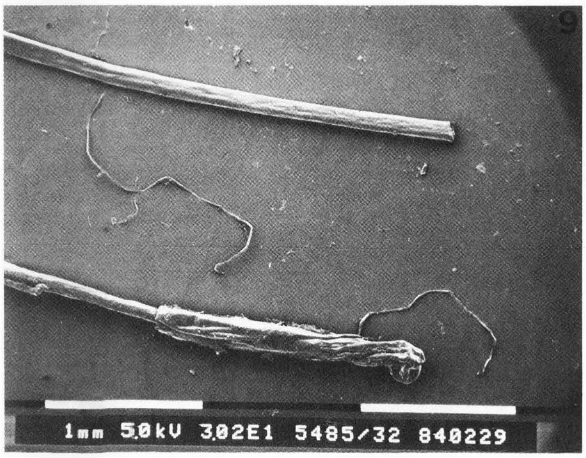
PIXE analysis : The fibre diameter of the analyzed hair was 58 µm and the conspicuous elliptiform cross-section may influence the mass determination and hence the quantitative elemental data. However, the Cu/Zn quotient which is 0.404 represents a remarkably high value (cf. case 8) compared to the normal value expected to lie around 0.1-0.2.

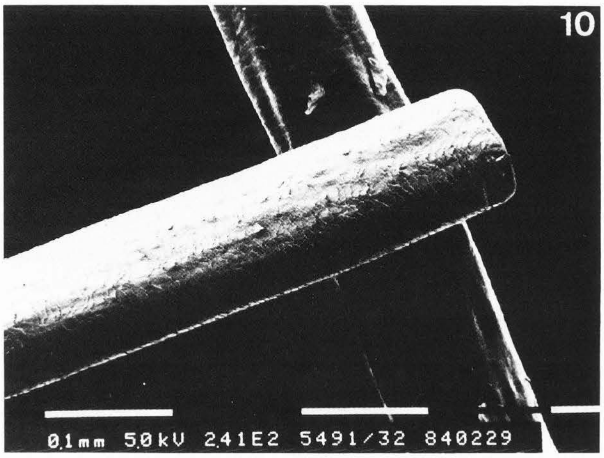
Case 8

Girl, born 1971. Sister of case 7. Tentative diagnosis: Epidermolysis bullosa Weber-Cockayne. Scalp hairs are clinically reported to appear normal in contrast to those of her









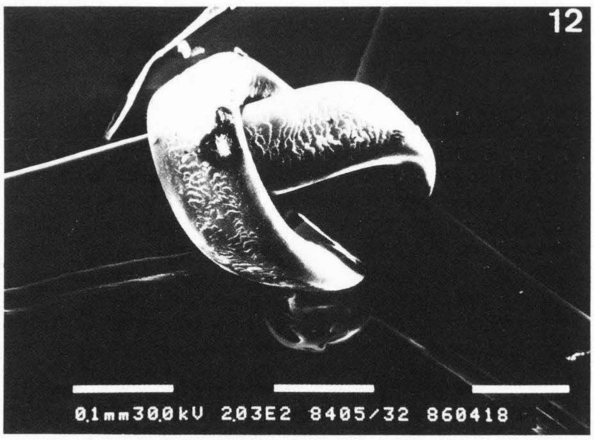


Fig 11: Case 6. Twisted hair fibre with obvious minor grooves (lower right). Bar = 1000 µm.

Fig 12: Girl 13 years old with tentative diagnosis epidermolysis bullosa Weber-Cockayne (case 7). Rather flat cross-section is demonstrated in the knot configuration. Cuticle pattern appears normal. Bar = 100 µm.

sister. In the SEM normal appearing hair fibres were seen.

PIXE analysis: In this case the analyses were duplicated on two different hair fibres obtained at the same occasion. The correspondence between the data is remarkably good. The fibre diameter in these cases was 74 µm in both specimens and an almost circular cross-section of the fibres should provide for a high accuracy in the determination of the mass and correspondingly of the elemental contents. A conspicuously high Cu/Zn quotient was observed.

Case 9

Girl, born 1981. Tentative clinical diagnosis: Spun glass hair. Hair on the scalp was sparse and apparently brittle since birth. The surface morphology revealed by the SEM varied a great deal. A number of fibres had more or less normal cross-sections, whereas other fibres were extremely flat (Fig 13). The cohesion of the cuticular cells appears diminished. The abnormal mechanical properties of these hair fibres were revealed by cutting the hair with a scalpel. This procedure caused a collapse of the fibre and resulted in a permanent flattening of the fibre at the cutting site (Fig 14).

Case 10

Girl, born 1980. Diagnosis: spun glass hair. No other anomalies are found. SEM revealed characteristic longitudinal grooves (pili canaliculi) (Fig 15), the irregular cross-section even producing rather sharp angles of the cross-section characteristic of this diagnosis. In a recently published paper the transmission electron microscopic (TEM) morphology of the cortex cross-section appeared completely normal (2).

PIXE analysis: The irregular cross-section may cause uncertainty in the mass determination. The data computed lay within the normal range.

Case 11

Girl, born 1981. Diagnosis: spun glass hair. No other anomalies are found. The SEM findings are identical to those of case 10 (cf. ref. 2).

PIXE analysis: The irregular cross-section of the hair may cause uncertainty in the mass determination. The data computed lay within the normal range.

Case 12

Boy, born 1979, with a history of an acrodermatitis-like condition caused by intestinal malabsorption. Treatment with zinc resulted in a conspicuous recovery from the skin involvement. The diagnosis is presently under reevaluation as it appears that the patient may suffer from a deficient prostaglandin synthesis and deficient absorption of linoleic acid influencing on the absorption of zinc. SEM investigation revealed normal looking hair fibres with rather ellipsoid cross-sections (Fig 16).

PIXE analysis: BT in table 1 denotes data obtained from a hair fibre plucked before substitution therapy with Zn; AT hair fibre plucked after several months of substitution therapy. These hair fibres are rather ellipsoid in cross-section as indicated by SEM and since they also are very thin, 43 µm in diameter, the uncertainty in the mass determination may

Fig 13: Girl 5 years old with tentative diagnosis Spun glass hair (case 9). The diagnosis incorrect in view of the abnormalities demonstrated in the SEM (cf. Fig 14). Hair fibre has the look of a flat shoe-lace and obviously lacks the normal rigidity as is demonstrated by the peculiar kink at the extreme left. Bar = 100 µm.

Fig 14: Same case as Fig 13. Cross cut of fibre causes collapse of the interior structure and displacement of loosely adhering cuticle scales. Bar = 10 µm.

Fig 15: A true Spun glass hair case (case 10). Longitudinal groove and irregular cross-section is clearly seen. Bar = 100 µm.

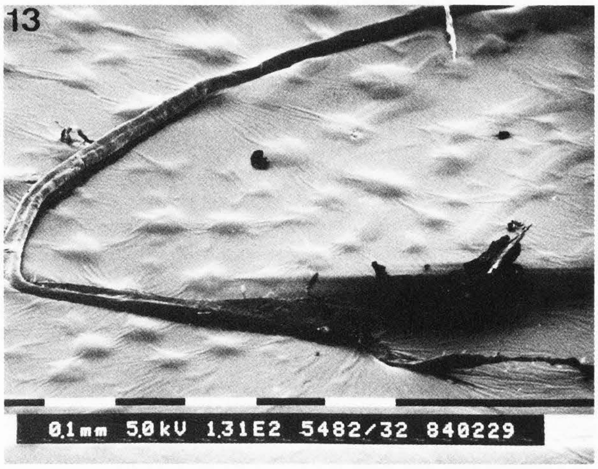
Fig 16: Boy 7 years old with history of acrodermatitis-like condition shown to be a case of malabsorption healed by zinc therapy. Normal configuration of hair with a slightly ellipsoidal cross-section. Bar = 100 µm.

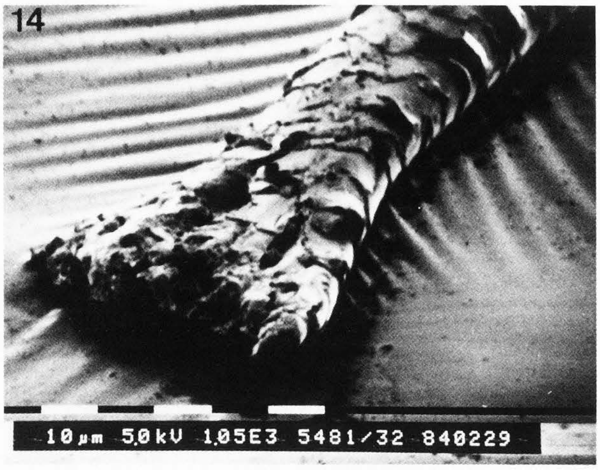
influence on the elemental data. However, the remarkable change in the S, Cu, and Zn data as a result of the substitution therapy were indeed paralleled with a complete recovery from the clinical symptoms.

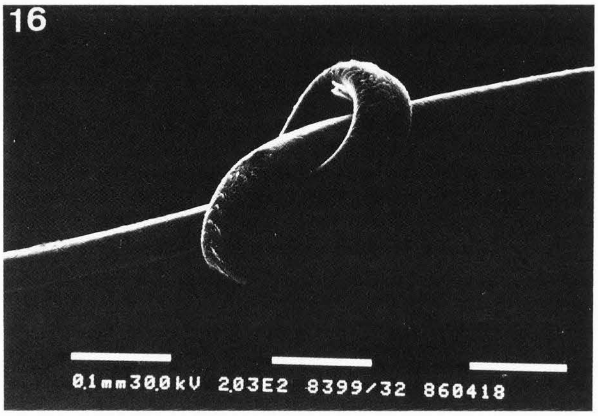
Discussion and Conclusion

The study of pathological hairs in the SEM allows the detection of abnormalities of the surface morphology but may also suggest and reveal the presence of defects deeper in the material under investigation. The cohesion of the cuticular cells may be tested by forming a knot on the hair fibre. When the cohesion is low, the cuticle 'shingles' stand out at an angle from the fibre axis; when the cuticular cells are abnormally soft as in Monilethrix (7) they smoothly adhere to the contour of the knot. In conditions with abnormal mechanical properties of the fibre, breaks may occur when forming the knot. Surface characteristics such as longitudinal grooves are seen in certain conditions, i.e., spun glass hairs (pili canaliculi). Although a number of pathological conditions have been described from a SEM point of view, still there is a need for collection of morphological data on particular characteristics that may be associated with defined clinical conditions. Unfortunately the combination of SEM and TEM investigations on the same material is extremely rare.

The PIXE analyses of the pathological hairs presented a number of problems. The sulfur values in general are low compared to those of the normal population (3). As was previously pointed out, the algorithm for the quantitative elemental analysis is based on a model with approximate circular cross-section and homogeneously distributed mass of the fibre cross-section. The very flat and twisted hair fibres of the present study may thus cause a relatively important uncertainty in the PIXE determination of the fibre cross-section which will influence on the mass determination and hence on the quantitative elemental data (5). These problems may, however, be circumvented by using the proton microprobe (PMP) on cross-sections as has been demonstrated previously (3). This method is







much more laborious as it requires semi-thin cryo-sections mounted on thin non-contributing plastic foils for analysis. Also, the time required per analysis is greatly increased. The cost/ benefit relation must therefore be analyzed before PMP is used routinely. However, it is also possible to compensate for irregularities in the fibre cross-section in the volume under analysis by using two detectors placed at right angles to each other.

With regard to the elements other than sulfur there is a considerable variation which may be due to contamination in the case of Cl, K, Ca, but probably reveal a true biological variation in Fe, Cu, and Zn. No clearcut pattern in the elemental contents can be discerned which may be related to a specific diagnosis in the cases reported here.

The coarse hair of the HCS (case 1 & 2) appear in the SEM as fibres with irregular cross-sections (Figs 1 & 2). There are no conspicuous characteristics that indicate any abnormality of the hair in these two cases. The PIXE analysis did not yield any data indicating abnormality.

Osteogenesis imperfecta is a disease with a disturbance of the sulfur metabolism which is expressed in an oversulfatation of proteoglycans. There are presently no data found in the literature to indicate disturbances in the hair follicle content and/ or distribution or metabolism, although the brittle hair of our case (case 3) suggests that this might be the case. The elemental PIXE analysis gives no clear-cut clue as the data obtained lie within the normal range (3). As discussed above, the very ellipsoidal cross-section of the fibres may be responsible for the failure of PIXE to reveal abnormal elemental contents and PMP analysis is therefore indicated.

Our two cases with unspecified diagnosis (cases 4 & 5) present SEM characteristics deviating from those of a normal hair fibre. However, these changes are of a more subtle nature and unspecific in relation to abnormal conditions. Diminished mechanical stability visualized by kinks formed on the fibre (Fig 7) makes it tempting to attribute this pathological behaviour to a low sulfur content, a fact born out only in case 4 at PIXE analysis. Other methods are therefore required. Case 5 as well as case 6 also deviate from the normal in the SEM without any specific and conclusive changes apparent. Also, the PIXE analysis is conclusive, apparently normal, but needs to be checked with other techniques for reasons discussed above.

The two sisters with the diagnosis epidermolysis bullosa Weber-Cockayne show hair surface structures which are normal-looking. The younger and more heavily inflicted sister (case 7) only shows a rather flat type of hair fibre in the SEM. In both cases 7 & 8 the Cu/ Zn quotient is so remarkably high that it must be ascribed to a truly abnormal Cu content. This is probably a reflection of the genetic disease in the two probands. The true 'spun glass hair' cases reported in the literature show no biochemical abnormalities in the hair and skin, the symptom being only localized to the hair. We have reported on two such cases (2) using SEM and TEM. In the present study PIXE analysis was performed but revealed no deviation from normal cases (cases 10 & 11). The irregular cross-section of the fibres may, however, require that PMP methods be used for the final analysis of elemental content.

A number of (genetic) diseases including rather unmanageable hair are given the diagnosis 'spun glass hair' from the ocular diagnosis. Case 9 is a representative of such cases. Here the SEM shows fibres with a conspicuously abnormal surface and mechanical characteristics not compatible with the tentative diagnosis (Figs 13 & 14). Unfortunately the hair mounts of this case were lost before PIXE analysis was performed.

Finally, case 12 is especially interesting as the malnutrition condition is reflected in the hair fibre analysis by PIXE before the Zn-substitution therapy (12BT in Table 1) where the Cu/ Zn quotient is high. Following Zn-substitution therapy the elemental contents resume normal levels (12AT in Table 1).

Hair is the tissue which normally contains the highest amounts of sulfur of all tissues, roughly 5% by weight. There are numerous studies on wool but also on human hairs to show that significant decreases in the sulfur content is paralleled with diminished mechanical stability of the hair fibres (cf. 10). In all genetic diseases a sulfur analysis is therefore pertinent. However, biochemical methods require access to substantial amounts of material. Physical methods such as the electron microprobe (EMP) or the proton microprobe (PMP and PIXE), however, allow sulfur determination from extremely small volumes (μl) and less. It is obvious from the data presented here that even the physical methods are hampered by the analysis conditions and most likely it is necessary to revert to PMP analysis of cross-sections of hair fibres to obtain reliable data in the future, when the cross section geometry endangers the accuracy of the PIXE method.

The obvious lack of patterns typical for specific conditions makes it necessary to compile data from a rather large set of individuals with a specified diagnosis to obtain a reference system which can aid the diagnosis in (genetic) diseases involving the integument.

At this preliminary state we conclude that SEM is a most valuable tool in the analysis of pathological hair fibres. The use of macro-PIXE in the analysis of such hairs involves a number of technical and computational problems which makes the interpretation of the quantitative data difficult. Most likely a microbeam method (PMP) is more suitable for the kind of problems dealt with in this study. However, this means that routine methods have to be abandoned for more laborious methods which undoubtedly will yield valuable detailed information on the elemental composition of the specimens.

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Discussion with Reviewers

Reviewer I: Does the Cu/Zn ratio have any physiological significance?
Authors: Many studies have indicated that the Zn level is remarkably constant in hair and that Zn may be used as an internal standard. In our previous study our results are in agreement with such a proposal (3). However, there is an age variation in the Zn concentrations before adolescence reported by Petering et al. (HG Petering, DW Yeager, SO Witherup (1971) Trace metal content of Hair. I. Zinc and copper content of human hair in relation to sex and age. Arch Environ Health 23, 202-207). Variations in Cu are much wider than those of Zn. Both elements are crucial as parts of different enzymes responsible for protein synthesis in cells. Thus Zn is responsible for a normal acting of mRNA on the polypeptide chain and Cu for the oxidative change of sulfydryl groups into disulfide bonds of the keratin. Generally there is a feeling that the Cu/ Zn ratio is more sensitive to physiologically significant changes than the absolute concentration of each element separately. If a single hair fibre is analyzed along its length, changes in the Cu/ Zn quotient are likely to indicate contamination.

Reviewer I: Are there any data about a systemic disturbance of Cu metabolism in epidermolysis bullosa Weber-Cockayne? Are serum or urine levels normal?
Authors: As far as we know the present victims of the Weber-Cockayne condition show no abnormalities at laboratory investigation.

S. Seta: What elements are responsible for the structural and compositional changes of genetic diseased hairs used in your study?
Authors: In most of our cases we have no clear indication of what element(s) may be involved in the cause of the abnormality. In the cases of epidermolysis bullosa Weber-Cockayne the high Cu and Zn data suggest a deficiency in the production of adequately functioning enzymes which is compensated with an increased production of the enzyme/ protein binding the trace element.